Effect of Inflammatory Cytokines on Hypoxia-Induced Erythropoietin Production

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The effects of the inflammatory cytokines interleukin-1α (IL-1α), IL-1β, IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) on erythropoietin (Epo) production in Hep3B cells were examined. The addition of IL-1α, IL-1β, or TNF-α resulted in a dose-dependent inhibition of hypoxia-induced Epo production by as much as 89%. IL-1β was the most effective cytokine tested, demonstrating half-maximal inhibition at 0.4 U/mL compared with 1.0 and 10.0 U/mL for IL-1α and TNF-α, respectively. TGF-β also inhibited hypoxia-induced Epo production, but only by as much as 56%. In contrast to IL-1α, IL-1β, TNF-α, and TGF-β, the addition of IL-6 to hypoxic Hep3B cells resulted in a dose-dependent stimulation of hypoxia-induced Epo production by as much as 81%. However, IL-6 did not stimulate Epo synthesis in the absence of hypoxia, and was thus synergistic with hypoxia in inducing Epo production. Combinations of IL-1α, TNF-α, and IL-6 were found to be additive in their effects on hypoxia-induced Epo production. By Northern blot analysis, Epo messenger RNA levels in Hep3B cells grown in 1% O_2 were decreased when concurrently exposed to either IL-1α or TNF-α. The effects that IL-1α, IL-1β, TGF-β, TNF-α, and IL-6 have on hypoxia-induced Epo production may provide new insights into the signal transduction pathway by which hypoxia leads to changes in gene expression. In addition, the effects of these inflammatory cytokines on hypoxia-induced Epo production in vitro suggest that in various inflammatory disorders these cytokines may affect Epo production in vivo and may play a significant role in the pathogenesis of the anemia of chronic disease.

ERYTHROPOIETIN (Epo) is a glycoprotein hormone crucial to the regulation of red blood cell production. It is produced by the liver[1] and the kidney[2] in response to hypoxia. In the fetus, the liver is the primary site of Epo production, whereas after birth the kidney becomes the major Epo-producing organ; however, in times of hypoxic stress the liver still contributes significantly.[3,4] In fact, using very accurate and sensitive ribonuclease protection assays, Tan et al[5] have recently shown that, with hypoxia, the liver in nonuremic rats contributes about 36% of total measurable Epo messenger RNA (mRNA), while in uremic animals the liver accounts for approximately 84% of total measurable Epo mRNA. Moreover, in anephric humans the liver is thought to be the main source of Epo production.[6] The hormone exerts its biologic effect by binding to specific receptors on erythroid progenitor cells, thereby inducing them to proliferate and differentiate into mature erythrocytes.[7,8] In addition to stimulation by hypoxia, Epo can also be stimulated by administration of cobalt.[9] The regulation of Epo gene expression occurs primarily at the mRNA level and is under the control of both transcriptional and posttranscriptional factors.[10,11] The level of Epo in the plasma ultimately influences the rate of production of new erythrocytes by the bone marrow. Failure to increase the amount of circulating Epo in response to hypoxic stress can lead to anemia.[12]

The anemia of chronic disease is typically a hypoproliferative anemia associated with low serum iron despite increased iron stores.[13,14] It is frequently encountered in such clinical settings as chronic infections, inflammation, and malignancy.[13] The pathogenesis of this anemia is still unclear, although it appears that the anemia of chronic disease may be the result of a multifactorial process that includes abnormal iron reutilization,[14,15] inappropriately low serum Epo levels for the degree of anemia, and a decreased bone marrow response to the Epo that is present. Steady-state serum Epo levels are frequently decreased for the degree of anemia in patients with a variety of infectious, inflammatory, and malignant disorders, including acquired immunodeficiency syndrome (AIDS), rheumatoid arthritis, ulcerative colitis, and cancer.[16-20] Furthermore, recent studies have shown that administration of recombinant human Epo can either partially or completely correct the anemia in patients with chronic diseases in which the Epo response is blunted,[21-26] strongly suggesting that a relative lack of endogenous Epo contributes to the pathogenesis of these anemias. This relative deficiency in the production of Epo is compounded by a diminished responsiveness of the bone marrow erythroid progenitor cells to Epo compared with normal bone marrow erythroid progenitors.[27]

The disorders associated with the anemia of chronic disease are characterized by the production of certain inflammatory cytokines, primarily macrophage-derived, including interleukin-1α (IL-1α), IL-1β, IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α).[13,28-33] In chronic inflammatory disorders, cellular or humoral factors, including TNF and IL-1, may cause suppression of the bone marrow response to Epo,[34-38] and thus possibly contribute to this anemia. To investigate the role of inflammatory cytokines in modulating the Epo response to anemia, we examined the effects in vitro of IL-1α, IL-1β, IL-6, TGF-β, and TNF-α on Epo protein production and on Epo mRNA levels. Because of the many confounding, indirect effects of in vivo manipulations, these experiments were performed using the human hepatoma cell line, Hep3B. While a kidney cell model would be ideal,

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after decades of searching, no model yet exists. The Hep3B cell line has been previously shown to regulate Epo production in a physiologic manner in response to hypoxia.[39,40] In fact, in all ways studied to date, it appears to reflect the physiologic situation in vivo, and it has been used by multiple investigators to study Epo gene regulation.[39-43] The Hep3B cell line is particularly appropriate for study of the effects of the inflammatory cytokines on Epo production because it has been shown to produce immunologically identifiable and biologically active Epo in a highly regulated manner in response to hypoxia or certain transition metals,[39,40] and it has also been used extensively as a model system for the effects of IL-1, TNF, and IL-6 in the acute phase response.[44-48] Previously, Jelkmann et al[49] reported that IL-1 and TNF-α reduce by approximately 50% the protein levels of Epo produced in HepG2 cells in response to hypoxia. In the present study, we use Hep3B rather than HepG2 cells because we have previously shown that the Hep3B cell line regulates Epo production much more definitively in response to hypoxia.[39,40] We show not only that IL-1 and TNF-α inhibit Epo production by up to 89%, but that TGF-β also has inhibitory effects that may differ from IL-1 and TNF-α. In addition, we present novel evidence that, in contrast to IL-1, TNF-α, and TGF-β, IL-6 in fact stimulates the production of Epo, and more importantly, that the net inhibition or stimulation of hypoxia-induced Epo production by Hep3B cells depends on the additive effect of the specific inflammatory cytokines present. This study thus provides a new approach to further investigate, at a cellular and molecular level, the nature of the molecular response to hypoxic stress.

MATERIALS AND METHODS

Cell culture. Hep3B cells were obtained through American Type Culture Collection (Rockville, MD). They were cultured in MEM-α medium (JRH Biosciences, Lenexa, KS) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% defined supplemented bovine calf serum (Hyclone, Logan, UT) and were maintained in a humidified 5% CO_2 incubator at 37°C. The cells were stimulated to produce Epo by growing them in the presence of $CoCl_2$ (75 μmol/L) or hypoxically in a controlled atmosphere chamber (Plas Labs, Lansing, MI) supplied with a constant flow of a hydrated 1% O_2, 5% CO_2, balance N_2 gas mixture. All experiments were begun when the Hep3B cells approached confluence.

In experiments in which incorporation of ^{3}H-uridine and ^{3}H-leucine into RNA and protein, respectively, were determined in Hep3B cells that were grown under hypoxic conditions, 25-cm^2 tissue culture flasks were fitted with rubber septa. The gas inlet and outlet were provided through 18-gauge needles. A gas mixture containing 1% O_2, 5% CO_2, and balance N_2 (Airco, Hingham, MA) was hydrated at 37°C and flowed at a constant rate into the tissue culture flasks. The flasks were placed in a 37°C incubator. pO_2 was monitored by collecting the outflow gas in a syringe and injecting the gas into an automated blood-gas analyzer (Instrumentation Laboratory Model 1304; Lexington, MA). ^{3}H-uridine and ^{3}H-leucine (New England Nuclear, Boston, MA) pulses were performed by injecting 1 mL of the appropriate ^{3}H-containing medium through the rubber septa, thereby keeping the cells hypoxic during the entire period of study.

Cytokine dose-response. Hep3B cells were incubated for 24 hours under hypoxic or normoxic conditions in triplicate with varying concentrations of recombinant human IL-1α (Boehringer Mannheim, Indianapolis, IN; 1,000 U/mL, specific activity, $>1 \times 10^7$ U/mg), IL-1β (Boehringer Mannheim; 1,000 U/mL, specific activity, $>1 \times 10^7$ U/mg), IL-6 (Boehringer, Mannheim; 200,000 U/mL, specific activity, $>2 \times 10^8$ U/mg), TNF-α (Boehringer Mannheim; 10 μg/mL with specific activity $>2 \times 10^7$ U/mg; thus, results expressed in units per milliliter represent a minimum estimate and assume a concentration of 200,000 U/mL), or TGF-β (gift from Dr David Scadden, New England Deaconess Hospital, Boston, MA). At the conclusion of each experiment, culture media was collected and frozen at −70°C until assayed by radioimmunoassay (RIA) for Epo and AFP.

RIA. The RIA for Epo was performed using a high-titer polyclonal rabbit antiserum raised against human recombinant Epo produced in our laboratory.[50] ^{125}I recombinant human Epo was obtained from Amersham, Inc (Arlington Heights, IL). Standards were prepared using recombinant human Epo (Amgen Inc, Thousand Oaks, CA) diluted in MEM-α medium containing 10% defined supplemented calf serum and 0.05% sodium azide, pH 7.4. Aliquots of 0.2 mL of standard or sample were placed in 5 mL conical polypropylene tubes. To this was added 0.1 mL of rabbit antiserum diluted 1:15,000 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. The mixture was diluted to 0.7 mL using the same diluent used for the recombinant Epo standards and was incubated at room temperature for 2 hours. ^{125}I Epo (0.1 mL; approximately 10,000 cpm) prepared in the same diluent was then added, and the mixture was briefly vortexed and incubated overnight at 4°C. Amerlex-M (1.0 mL; Donkey anti-rabbit globulin; Amersham) was then added to each tube and the tubes were placed at 4°C with constant shaking for 2 hours. The Amerlex was pelleted by centrifugation for 15 minutes at 1,500g at 4°C, washed once with 1.0 mL PBS, and counted in an LKB model 1282 gamma counter (Pharmacia LKB Nuclear, Inc, Gaithersburg, MD).

Alpha-fetoprotein (AFP) was quantitated in media samples using an AFP/OB Radioimmunoassay Kit (Kallestad Diagnostics, Chaska, MN). This RIA uses ^{125}I-labelled AFP, and is accurate within a range of 7.5 to 180 IU/mL.

Biosynthetic labeling. Experiments were performed to assess the effects of a 1% versus 21% O_2 environment on total RNA and protein synthesis by Hep3B cells, as well as the effects of IL-1β, TNFα, and IL-6 on RNA and protein synthesis in Hep3B cells grown in 1% O_2. After incubation in the desired oxygen environment with the desired cytokines, cells were pulsed for 1.5 hours with media containing either 10 μCi of ^{3}H-uridine or 40 μCi of ^{3}H-leucine. The media was then removed, the cells solubilized in 0.1 N NaOH/0.25% deoxycholate, and precipitated with ice-cold 15% trichloroacetic acid (TCA). The TCA precipitable counts were measured using an LKB model 1219 scintillation counter. The corresponding Epo production was determined by performing an Epo RIA on aliquots of the media in which the cells were grown.

Northern blot analysis. Total RNA was prepared from cultured cells as described by Chirgwin et al.[51] The RNA was denatured in formaldehyde, electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde and a trace amount of ethidium bromide, and transferred to a GeneScreen Plus filter (New England Nuclear) using 10× standard saline citrate (1.5 mol/L NaCl. 0.15 mol/L sodium citrate).[52] Epo cDNA in an SP65 plasmid (gift from Dr Charles Shoemaker, Genetics Institute, Inc, Cambridge, MA) was digested with the restriction enzyme *Eco*RI, the Epo insert isolated by agarose gel electrophoresis, followed by electroelution[52] and ^{32}P-labeled to a specific activity of between 3×10^8 and 1.2×10^9 cpm/μg of cDNA.[53] The radiolabeled cDNA was then mixed with carrier salmon sperm DNA, denatured by boiling for 10 minutes, and hybridized to the filter at 5×10^5 cpm/mL of hybridization

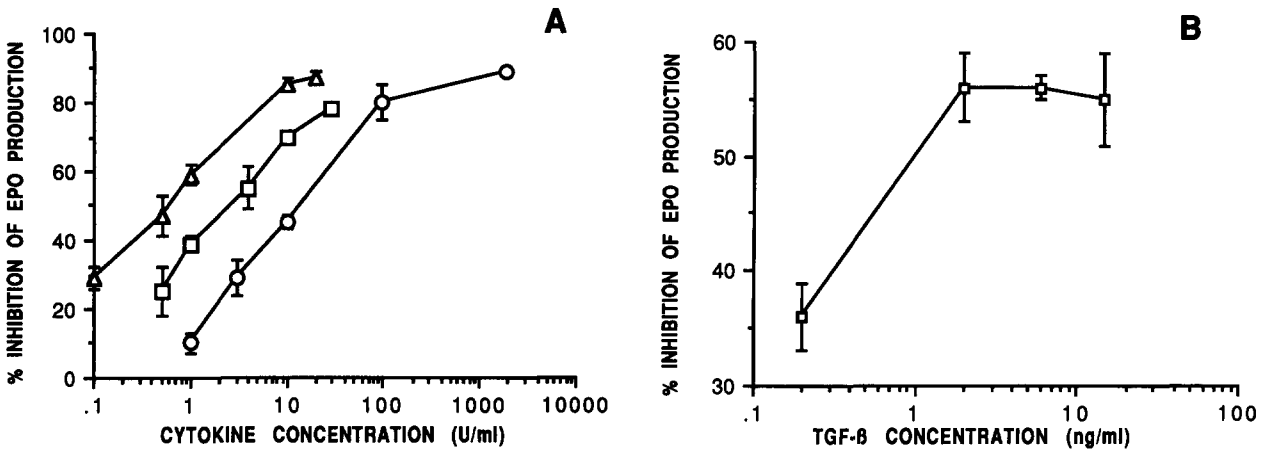

Fig 1. Dose-response inhibition of hypoxia-induced Epo production by IL-1α, IL-1β, TNF-α, and TGF-β. Hep3B cells were grown to confluency in 100-mm tissue culture dishes and incubated under hypoxic conditions (1% O_2) for 24 hours in triplicate with varying concentrations of (A) IL-1α (□), IL-1β (△), TNF-α (○), or (B) TGF-β. At the conclusion of each experiment, the culture medium was collected and stored at −70°C until assayed in duplicate by RIA for Epo. Values shown represent the mean percent inhibition of hypoxia-induced Epo production ±1 SD.

solution (50% formamide, 1 mol/L NaCl, 1% sodium dodecyl sulfate [SDS], 10% dextran). Hybridization was performed at 42°C for 20 hours. The final washing was in 0.5× standard saline citrate (0.075 mol/L NaCl, 0.0075 mol/L sodium citrate) at 65°C. Relative amounts of radiolabeled cDNA that hybridized to the blots were quantitated using a Betascope 603 Blot Analyzer (Betagen Inc, Waltham, MA). Autoradiography was performed with intensifying screens at −80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY). Mouse β-actin cDNA was also radiolabeled and hybridized to the same filters to provide an internal control for the efficiency of RNA transfer to the filters. Relative levels of Epo mRNA were normalized to β-actin mRNA levels as measured by the Betascope.

RESULTS

Hep3B cells grown in 1% O_2 for 24 hours typically produced approximately 50- to 100-fold more Epo compared with cells grown under comparable conditions in 21% O_2. For example, in the experiments depicted in Fig 1A and B, the mean (±1 SD) Epo concentration in the medium after 24 hours at 1% O_2 was 195 ± 22 mU/mL (n = 9 plates) compared with 2 ± 7 mU/mL (n = 9 plates) for control cells grown at 21% O_2 for the same period of time. The effect of inflammatory cytokines on Epo production was investigated using Hep3B cells incubated under hypoxic conditions (1% oxygen) with varying concentrations of IL-1α, IL-1β, IL-6, TGF-β, and TNF-α (Figs 1 and 2). IL-1α, IL-1β, and TNF-α inhibited hypoxia-induced Epo production by up to 89% in a dose-dependent manner (Fig 1A). IL-1β was the most potent cytokine tested for inhibition of Epo production with half-maximal inhibition at 0.4 U/mL compared with 1.0 and 10.0 U/mL for IL-1α and TNF-α, respectively. TGF-β also decreased Epo production by hypoxic Hep3B cells; however, unlike IL-1α, IL-1β, and TNF-α, TGF-β's maximal dose-dependent inhibition of hypoxia-induced Epo production was only 56% (Fig 1B). In contrast with IL-1α, IL-1β, TGF-β, and TNF-α, the addition of IL-6 to hypoxically stimulated Hep3B cells caused a dose-dependent increase of up to 81% in Epo production with half-maximal stimulation at 250 U/mL (Fig 2). IL-6 did not stimulate Epo production under normoxic conditions, and thus its effect is considered to be synergistic with hypoxia.

When tested in combination, the effect of the inflammatory cytokines on hypoxia-induced Epo production was approximately additive (Fig 3). Alone, IL-1α (0.35 U/mL) and TNF-α (2.5 U/mL) decreased Epo by 21% ± 5% and 15% ± 1%, respectively, whereas together they inhibited Epo by 41% ± 2% (Fig 3). Similarly, IL-6 (2,000 U/mL), which alone stimulated hypoxia-induced Epo by 81% ± 8%, was able to reverse the above-mentioned 41% IL-1α + TNF-α–induced decrease in Epo production to a 41% increase in Epo production. IL-6 was also additive in combination with either IL-1α or TNF-α alone (data not shown).

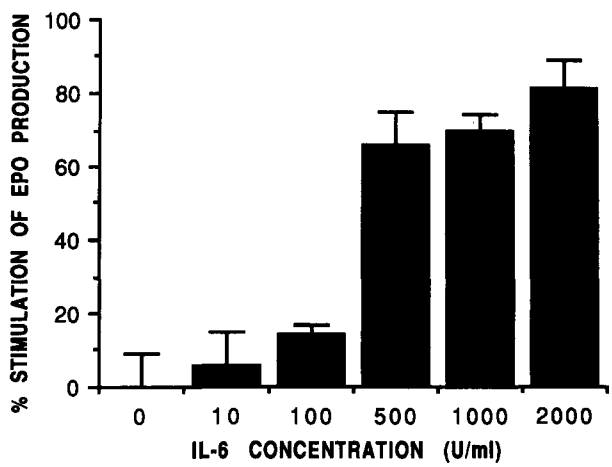

Fig 2. Dose-response stimulation of hypoxia-induced Epo production by IL-6. Confluent 100-mm plates of Hep3B cells were incubated in triplicate for 24 hours under hypoxic conditions with varying concentrations of IL-6. Culture media was collected and assayed in duplicate by RIA for Epo. Values represent the mean percent stimulation of hypoxia-induced Epo production above that produced by hypoxia alone ±1 SD.

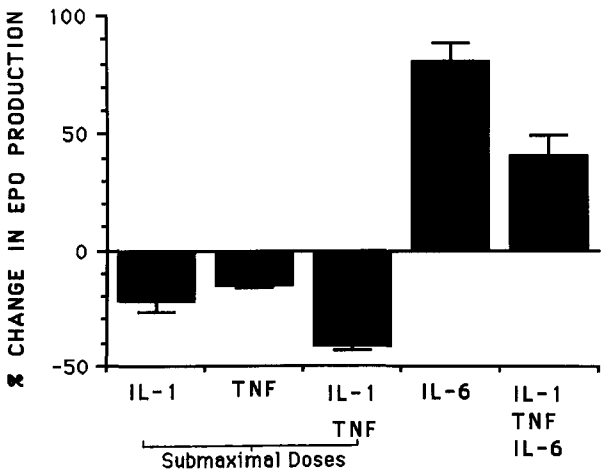

Fig 3. Additive effects of IL-1α, TNF-α, and IL-6. Confluent 100-mm plates of Hep3B cells were incubated in triplicate for 24 hours under hypoxia in the presence of IL-1α (0.35 U/mL), TNF-α (2.5 U/mL), IL-1α + TNF-α, IL-6 (2,000 U/mL), or IL-1α + TNF-α + IL-6 as indicated. Media was collected and assayed in duplicate RIA for Epo. Values are the percent change in Epo production relative to hypoxia alone ±1 SD.

The relative specificity of the IL-1α, IL-1β, IL-6, TGF-β, and TNF-α effects on Epo production were assessed in three independent ways. Samples of the growth media were assayed for AFP (Fig 4). We observed at most a 20% variation in AFP production that was not dose-dependent. In addition, because cobalt chloride has previously been shown to stimulate Epo production independently of hypoxia, the effects of IL-1α, TNF-α, and IL-6 on cobalt chloride-induced Epo production were measured. The various cytokines were found to have similar effects on both cobalt- and hypoxia-stimulated Epo production (data not shown).

The effect of the inflammatory cytokines on Epo mRNA levels was examined using Northern blot analysis (Fig 5). Relative to the amount of Epo mRNA produced under normoxic conditions (21% O_2) and normalizing to β-actin mRNA levels, we observed approximately a 75-fold increase in mRNA in 1% O_2 that was reduced in the presence of IL-1α (20 U/mL) or TNF-α (1,000 U/mL) to 25- and 10-fold increases, respectively, above that seen in 21% O_2 (Fig 5). Furthermore, addition of IL-6 (1,000 U/mL) to hypoxic Hep3B cells resulted in greater than a 85-fold increase in Epo mRNA relative to normoxia. In this experiment, the amount of Epo produced by Hep3B cells treated with IL-1α, TNF-α, or IL-6 was measured by RIA; IL-1α and TNF-α inhibited hypoxia-induced Epo production by 81% and 90%, respectively, whereas IL-6 increased hypoxia-induced Epo production by an additional 59%.

Biosynthetic labeling experiments were performed with ^{3}H-uridine and ^{3}H-leucine in Hep3B cell grown in 1% versus 21% O_2 and in 1% O_2 in the presence and absence of IL-1β, TNFα, and IL-6. These experiments, the results of which are shown in Table 1, demonstrated that total RNA synthesis decreased approximately 85% and total protein synthesis decreased approximately 47% when Hep3B cells were grown in 1% O_2 compared with 21% O_2. However, IL-1β, TNF-α, and IL-6 increased total RNA synthesis and had no significant effect on protein synthesis in Hep3B cells grown in 1% O_2. In these biosynthetic labeling experiments, Epo levels in the media were concurrently measured and were consistent with the results of previous experiments shown in Figs 1A and 2. These results demonstrate that the inhibitory effects of IL-1 and TNF-α on hypoxia-induced Epo production cannot be toxic or nonspecific effects.

DISCUSSION

In these experiments, Hep3B cells grown in 1% O_2 typically increased Epo production by 50- to 100-fold in a 24-hour period compared with cells grown in 21% O_2. This degree of hypoxia-induced stimulation of Epo production is much greater than the approximately threefold stimulation that we and others have observed using the HepG2 cell line.[39,49] This greater stimulation of Epo production by Hep3B cells more closely mimics the physiologic response to hypoxia, and permits a much more accurate assessment of agents that might alter this response.

These results demonstrate that the inflammatory cytokines IL-1α, IL-1β, TGF-β, TNF-α, and IL-6 are able to modulate the production of Epo by hypoxia- or cobalt chloride-stimulated Hep3B cells. IL-1α, IL-1β, TGF-β, and TNF-α all caused a dose-dependent inhibition of Epo production (Fig 1), whereas IL-6 caused a dose-dependent stimulation (Fig 2). It is noteworthy that IL-1 and IL-6 have opposite effects on hypoxia-induced Epo production because in most acute phase responses described previously, these two cytokines have synergistic effects.[28,29,54,55] The lack of a dose-dependent change in AFP levels (Fig 4), and the lack of an effect on β-actin mRNA levels (Fig 5), as well as the minimal effect on overall protein synthesis and stimulatory effect on total RNA synthesis (Table 1), all indicate that the observed effects of the inflammatory cytokines on Epo production are specific. In addition, Northern blot analysis shows that, at least in the case of IL-1α and TNF-α,

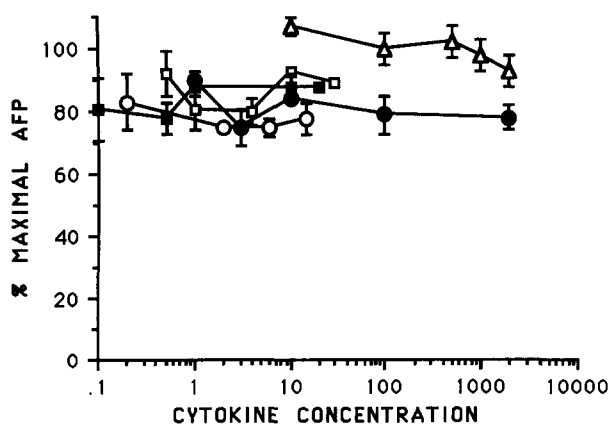

Fig 4. Lack of effect of inflammatory cytokines on levels of AFP. Media obtained from confluent plates of Hep3B cells that had been incubated for 24 hours under hypoxia with varying concentrations of IL-1α (□), IL-1β (■), IL-6 (△), TGF-β (○), or TNF-α (●) was assayed in duplicate by RIA for AFP. Values represent the mean amounts of AFP relative to hypoxia alone ±1 SD, and are plotted in units per milliliter except for TGF-β, which is plotted in nanograms per milliliter.

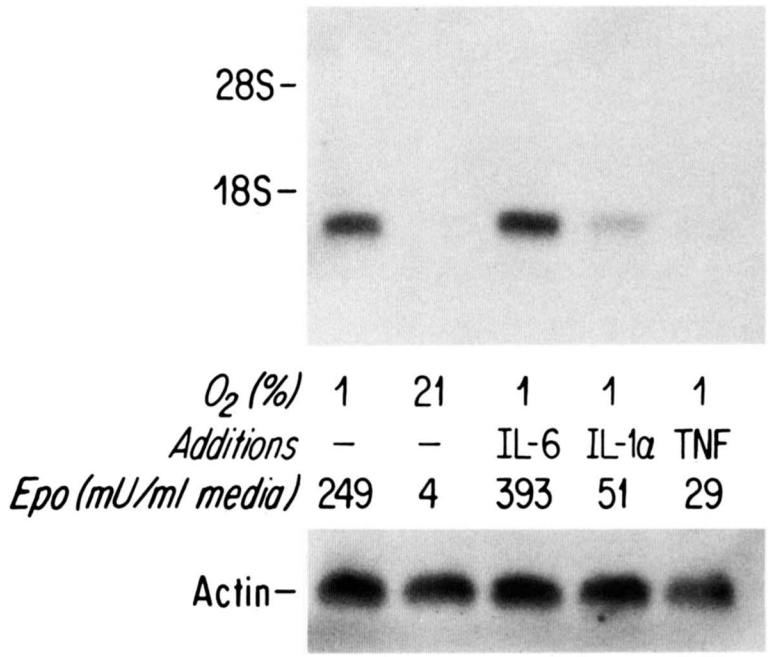

Fig 5. Effect of IL-6, IL-1α, and TNF-α on hypoxia-induced Epo mRNA levels. Hep3B cells grown to confluency in 150-mm plates were incubated either nonhypoxically (21% O_2) or hypoxically (1% O_2) for 24 hours in the presence or absence of IL-6 (1,000 U/mL), IL-1α (20 U/mL), or TNF-α (1,000 U/mL) as indicated. Total cellular RNA was isolated and RNA blot analysis was performed using 20 μg total RNA per lane. The results obtained when the RNA-containing filter was hybridized with ^{32}P-labeled Epo cDNA are shown at the top. As a control, the hybridization to radiolabeled mouse β-actin is presented at the bottom. In addition, the amount of Epo produced by the cells was determined in duplicate by RIA as shown.

the changes observed in Epo protein production result from changes in the levels of Epo mRNA (Fig 5). The level at which the stimulatory effect of IL-6 on hypoxia-induced Epo production occurs is less clear. While the data show a modest increase in Epo mRNA levels when hypoxically grown Hep3B cells are exposed to IL-6, ^{3}H-uridine pulse-labeling studies show increases in overall RNA synthesis to a similar degree.

Because Hep3B cells produce Epo as well as acute phase proteins in response to normal physiologic stimuli,[39,40,44-48] they serve as a good in vitro model for both the study of Epo regulation and the acute phase response. The specific effects of the inflammatory cytokines on Epo production in Hep3B cells suggest that these mediators may also be important regulators of Epo production in vivo. As combinations of IL-1α, TNF-α, and IL-6 were found to be additive in vitro (Fig 3), it is possible that, in vivo, the relative production of one cytokine versus another could result in either down regulation or upregulation of Epo. This might explain the seemingly aberrant Epo levels observed in patients with various inflammatory disorders.[16-20] However, while our results indicate that the inflammatory cytokines can modulate the hypoxia-induced production of Epo in a hepatoma-derived cell line, it is not known if the inflammatory cytokines will have the same effects on Epo production in the kidney. In fact, recent experiments in transgenic mice suggest that differences may exist between the regulation of Epo expression in liver versus kidney.[43] However, the transgenic model is limited by variations and differences in gene expression from one transgenic strain to the next, depending on, at least in part, the particular chromosomal integration site of the trans gene. More recently, Jelkmann et al have found that IL-1β significantly inhibits Epo production in the hypoxic, isolated perfused kidney (W. Jelkmann, personal communication, August 1991), thus suggesting that the inflammatory cytokines may indeed play an in vivo role in modulating both liver and kidney Epo production. Studies are now being planned to see if we can extend our results to an in vivo animal model of the anemia of chronic disease.

The fact that the levels of cytokines used in these studies to inhibit hypoxia-induced Epo production in Hep3B cells were within the range of cytokine plasma levels reported in several chronic disorders suggests that the inflammatory cytokines could play a role in the anemia of chronic disease. Normally, the levels of IL-6 in the blood are reported to be less than 0.09 ng/mL (approximately 20 U/mL); however, under some conditions, such as during the stress of infection, in endotoxemia, and in some patients undergoing

Table 1. Biosynthetic Labeling Experiments

Experiment No.	Experimental Conditions	^{3}H-Leucine Precipitable Counts (cpm)	^{3}H-Uridine Precipitable Counts (cpm)	Epo (mU/mL)
1	21% O_2	49,700		1
	1% O_2	26,200		90
	1% O_2 + IL-1β (10 U/mL)	27,100		12
	1% O_2 + TNF-α (100 U/mL)	25,900		12
	1% O_2 + IL-6 (1,000 U/mL)	28,700		147
2	21% O_2		5,900	2
	1% O_2		900	84
3	1% O_2		1,500	130
	1% O_2 + IL-1β (10 U/mL)		4,300	20
	1% O_2 + TNF-α (100 U/mL)		3,300	30
	1% O_2 + IL-6 (1,000 U/mL)		2,700	200

In experiments one and two, the tritiated labeling was begun after 15.5 hours, and in experiment three the labeling was begun after 21.5 hours exposure to the relevant experimental conditions. Each experimental condition was performed in duplicate or triplicate and the table shows the mean of the TCA-precipitable counts and the Epo concentrations.

renal transplantation the concentration of IL-6 has been shown to increase by as much as 2,000-fold.[56-60] In addition, consistent with our finding that IL-6 increases the hypoxia-induced production of Epo, elevated serum Epo levels and an erythrocytosis have been described for some cases of renal transplantation.[61,62] TNF-α, which is normally present in serum at less than 9 U/mL, has also been reported to be elevated in various chronic conditions, including inflammatory and infectious disorders where TNF-α levels can reach as high as 600 U/mL and in chronic heart failure where TNF-α can reach 115 U/mL.[31,33,60,63] Similarly, in over 50% of cancer patients with active disease and in whom there is frequently an associated anemia with a blunted Epo response, the levels of TNF-α in the serum are significantly elevated.[64]

The observation that IL-6 is synergistic with hypoxia in stimulating Epo production in Hep3B cells raises the possibility that IL-6 might have an in vivo role in the treatment of various anemias. Certainly the Hep3B model is much simpler than the in vivo situation, and any extrapolation would be pure speculation. However, several in vivo studies of the effects of IL-6 in murine models have been performed.[65-67] IL-6 has been noted to cause an early reticulocytosis and erythroid hyperplasia of the bone marrow when administered as a single intravenous bolus.[65] A subsequent study by this same group,[67] in which human IL-6 was administered by daily intravenous injection, found the erythropoietic effects to be of a transient nature and suggested that it was not caused by stimulation of endogenous Epo. Of note, in this study the animals were not anemic, and, hence, presumably not hypoxic; because IL-6 was only synergistic with hypoxia in stimulating Epo production in Hep3B cells, these in vivo findings are not inconsistent with our findings. In contrast, Brandt et al[66] showed that dysregulated IL-6 expression resulted in an anemia, transient granulocytosis, splenomegaly, and lymphadenopathy in congenitally anemic W/W^V mice reconstituted with hematopoietic cells infected with a retroviral vector expressing murine IL-6. Clearly, further studies are needed to clarify the in vivo role of IL-6 as a modulator of Epo production.

The mechanisms by which the inflammatory cytokines affect Epo production in Hep3B cells are unclear. There are several points in the Epo regulatory pathway where the inflammatory cytokines could act to change Epo mRNA levels and thereby alter Epo production.[10,11] Changes in Epo production could occur by direct effects on Epo transcription, or by effects on Epo mRNA stability. The similarities in the maximal achievable inhibition, and in the slopes of the dose-response curves for IL-1α, IL-1β, and TNF-α (Fig 1A), suggest that these three cytokines possibly act through a similar inhibitory pathway. Recently, we observed that phorbol esters also inhibit Epo production by Hep3B cells.[68] This raises the possibility that the inflammatory cytokines may modulate Epo production via a protein kinase C-mediated pathway. Furthermore, a sequence (TTGCGGAAC) similar to the concensus sequence for NFIL-6, a nuclear factor involved in the expression of genes regulated by IL-6,[69] is present in the 5′ flanking region of the Epo gene. Further study of this region, located approximately 366 bp upstream of the putative CAP site of the Epo gene, may help to elucidate the pathway involved in the observed IL-6–mediated upregulation of hypoxia-induced Epo production.

Understanding the influence of the inflammatory cytokines on Epo production hopefully will clarify the interplay of the multiple factors involved in the pathogenesis of the anemia of chronic disease. However, of even greater interest, is the potential to use these cytokines to gain insight into the cellular and molecular responses to an hypoxic stimulus. Oxygen differs from the great majority of external cell signals by being a ubiquitous molecule that diffuses readily into cells independent of receptor binding. Accordingly, the signal transduction pathway from oxygen sensor to gene regulation may differ significantly from those associated with classic ligand-receptor binding. Investigation of the molecular basis for the observed effects of these inflammatory cytokines on hypoxia-induced Epo production should provide detailed information on the molecular nature of the oxygen sensor. A better understanding of the molecular mechanisms governing the response to hypoxic stress may have potential applications to many areas of medicine.

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